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Comparison of Sterols of Pollens, Honeybee Workers, and Prepupae From Field Sites

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Sterols from pollen collected by foraging honeybees, *Apis mellifera* L, at seven field sites were compared with the sterols of foraging adults and/or prepupae collected from colonies at each site. Invariably, the composition of prepupal sterols was comparable to that found in previous cage studies using chemically defined diets containing various dietary sterols: 24-methylenecholesterol was the major sterol; sitosterol and isofucosterol were present in lesser, but significant amounts; and a trace amount of cholesterol was identified in each sample. This occurred even though some of the pollen sterols contained little 24-methylenecholesterol, sitosterol, or isofucosterol and a preponderance of certain other sterols, such as Δ^7-stigmasten-3β-ol and $\Delta^{7,24(28)}$-campestadien-3β-ol in goldenrod and corn pollens, respectively. Thus the selective transfer and utilization of sterols in honeybees that have been demonstrated in cage studies with artificial diets were also shown to occur under field conditions.

Key words: honeybees, pollens, sterols, field sites

INTRODUCTION

In previous studies on the utilization and metabolism of sterols in the honeybee, *Apis mellifera* L, we determined that honeybees cannot dealkylate C_{28} or C_{29} phytosterols at the C-24 position to produce cholesterol or other C_{27} sterols [1, 2], as most phytophagous insects can [3], or convert C_{28} or C_{29} phytosterols to 24-methylenecholesterol [1]. We also demonstrated that adult honeybees possess a selective mechanism that enables them to provide certain sterols (mainly 24-methylenecholesterol, sitosterol, and isofucosterol) from their endogenous pools, in significant amounts to the developing brood, regardless of the dietary sterol available to the workers [4]. Studies in which various dietary sterols were added to the worker diet revealed that 24-

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methylenecholesterol was always the major component of the sterols isolated from the brood prepupae, and more recently we found that radiolabeled 24-methylenecholesterol, administered either by feeding or injection, was passed unchanged through a second generation of honeybees [2]. In addition, fairly consistent amounts of sitosterol and isofucosterol, as well as small but identifiable amounts of cholesterol and desmosterol, accompanied 24-methylenecholesterol in the prepupal sterols.

Our previous metabolism studies consisted of cage experiments in which a well-defined artificial diet was supplemented with highly purified sterols [5]. To determine whether the process of cycling certain sterols from endogenous pools of the worker bees into the brood food could be demonstrated with foraging colonies in the field, we compared the sterol composition of pollen samples, foraging adult honeybees, and prepupae collected at seven field sites chosen because the major source of pollen differed at each site. We report here that the results of these analyses of field-collected samples substantiate that the mechanism responsible for maintaining the apparent critical mixture of sterols in developing honeybee brood is similar whether bees function under field conditions or controlled cage conditions.

MATERIALS AND METHODS

Field Collected Samples

Colonies were established at seven sites in Beltsville, Maryland, with one 3-pound package of Italian bees plus a mated laying queen obtained from a commercial source (York Bee Co, Jesup, Georgia 31545) per colony.* Pollen traps were used to collect pollen samples (17–35 g/sample) at each location, and the pollens were identified by microscopic examination. It was determined that the foraging workers at each site visited primarily a single pollen source. The principal pollen sources (and their family names) at the sites were as follows: 1) maple (Aceraceae), 2) blackberry (Rosaceae), 3) cucumber (Cucurbitaceae), 4) goldenrod (Compositae), 5) corn (Gramineae), 6) honeysuckle (Caprifoliaceae), and 7) dandelion (Compositae). A small amount of other pollens was present in all samples, but the dandelion pollen sample was the most impure, containing about 40% of other pollens (Cornaceae and Rosaceae). Prepupae (25 per sample) from the first cycle of brood and foraging adults (25 per sample) were collected from all but the honeysuckle and dandelion colonies. Pollen adhering to adults was removed when they were collected prior to storage. All samples were weighed and stored frozen until processing for sterol isolation.

Isolation and Purification of Sterols

Pollen samples (17–35g) were saponified under reflux for 4 h in 160 ml EtOH, 20 ml benzene, 20 ml H_2O, plus 5% KOH. The mixture was cooled, 200 ml of H_2O was added, and the solution was acidified with 1 N HCl, and extracted 4× with hexane and 1× with Et_2O. The organic solvent phases were combined, dried over Na_2SO_4, and filtered. After the solvent was

*Mention of a company name or proprietary product does not constitute an endorsement by the US Department of Agriculture.

removed in vacuo, the sterols were isolated by column chromatography on alumina (Woelm, ICN Pharmaceuticals, Cleveland) as previously described [4,6]. Column fractions were monitored by thin-layer chromatography.[†]

Adult and prepupal samples were homogenized in $CHCl_3$-MeOH (2:1) with a VirTis homogenizer. The crude lipids were saponified, and the sterols in the nonsaponifiables were isolated by column chromatography in the same manner as the pollen samples. Subsequently, all pollen, prepupal, and adult sterol samples were precipitated through the digitonide to remove fatty alcohols and other impurities prior to gas-liquid chromatography.

Analysis of Sterols

Sterol samples were analyzed quantitatively and qualitatively by GLC on three systems: 2% OV-17 coated on Gas Chrom P (100–120 mesh) packed in a 1.8 m × 2 mm ID column, at 249°C; J & W DB-1 fused silica capillary column, 15 m × 0.25 μm film, at 219°C, helium carrier gas at 25 cm/sec linear velocity, 23:1 split ratio; 2% SE-30 coated on Gas Chrom P (100–120 mesh) packed in a 1.8 m × 4 mm ID column at 230°C. The first two columns were in Varian model 3700 gas chromatographs and the third was in a Hewlett-Packard model 7610A gas chromatograph. Sterol identifications by GLC were based on comparisons of relative retention times with cholestane as internal standard. Identities of all sterol samples were established by gas chromatography–mass spectrometry with a Finnigan model 4510 automated GC-MS equipped with a J & W DB-1 fused silica capillary column, 15 m × 0.32 mm (0.25 μm film). Proton magnetic resonance spectra were recorded with a JEOL FX-60-Q Fourier Transform NMR equipped with a 1.7 mm capillary probe. Infrared spectra were run on a Perkin-Elmer model 221 infrared spectrophotometer.

Portions of all pollen sterol samples were acetylated (in pyridine-acetic anhydride, 2:1, room temperature, overnight), and the acetates were examined by GLC and GC-MS. The acetates were then fractionated by argentation chromatography on 20% $AgNO_3$-impregnated Unisil as previously described [7], and column fractions were monitored on $AgNO_3$-impregnated silica gel H chromatoplates.

RESULTS

Most sterol samples of pollen, honeybee adults, and prepupae were easily identified, but GLC and GC-MS analyses indicated that the major sterol of both the goldenrod and corn pollen samples differed from that in any of the other pollens. The major sterol from goldenrod pollen gave an IR spectrum with absorption bands in the 800–850 cm^{-1} region typical of a Δ^7-sterol. The GC-MS spectrum of the sterol acetate, showing a strong molecular ion at m/z 456, was identical to that of authentic Δ^7-stigmasten-3β-ol acetate. The RRTs of the free sterol and its acetate corresponded to those of authentic Δ^7-stigmasten-3β-ol and its acetate, respectively, on all three GLC systems. The

[†]Abbreviations: gas chromatography-mass spectrometry = GC-MS; gas liquid chromatography = GLC; proton magnetic resonance = PMR; relative retention times = RRTs; thin-layer chromatography = TLC.

R_f values for the acetates on an $AgNO_3$-impregnated TLC plate were also identical.

The major sterol of corn pollen on the capillary GLC system had an RRT between the RRTs of stigmasterol and Δ^7-campestenol. Its acetate was somewhat less polar than 24-methylenecholesterol acetate on an $AgNO_3$-TLC plate. The IR spectrum of the acetate showed a strong peak at 892 cm^{-1}, indicative of a di-substituted ethylene. The PMR spectrum showed olefinic proton peaks centered at δ 5.13 and δ 4.74, representing one and two protons, indicating a tri-substituted and a di-substituted ethylene, respectively. The GC-MS of the sterol acetate showed a molecular ion at m/z 440 and other peaks at m/z 425 ($M-CH_3$), 380 ($M-CH_3COOH$), 356 ($M-C_6H_{12}$) resulting from fragmentation of the 22–23 bond coupled with a hydrogen transfer, 365 ($M-CH_3COOH-CH_3$) and base peak at m/z 313 ($M-C_9H_{19}$), which resulted from cleavage of the 17–20 bond accompanied by a transfer of two hydrogens from the sterol nucleus [8]. Such a strong cleavage is typical of Δ^7-sterols [8]. The mass spectrum of the free sterol also showed this cleavage, which resulted in the base peak at m/z 271. The peak at m/z 314 also indicates a cleavage of the 22–23 bond. The foregoing results suggested that this sterol was $\Delta^{7,24(28)}$-campestadien-3β-ol.

Subsequently, both the free sterol and its acetate were isomerized and hydrogenated in the presence of acetic acid and 10% palladium on charcoal to $\Delta^{8(14)}$-campesten-3β-ol and its acetate, respectively. Thus, this indicates that a Δ^7-bond had isomerized to the $\Delta^{8(14)}$ position and the $\Delta^{24(28)}$-bond had been reduced. The results of mass spectral and GLC analyses of these hydrogenation products were identical with those of the authentic sterol and sterol acetate. These results established that the major sterol from this corn pollen sample was $\Delta^{7,24(28)}$-campestadien-3β-ol.

The sterol contents of pollen, honeybee adult, and prepupal samples analyzed in this study are summarized in Table 1. Some minor sterols, particularly in the pollen samples, that are not of significance to this discussion are not included. Since cholesterol, desmosterol, 24-methylenecholesterol, sitosterol, and isofucosterol were common to all honeybee samples we had previously examined [1,4], data for these five sterols are included for each sample in Table 1.

24-Methylenecholesterol was only a minor sterol in several of the pollen samples. This is particularly true in the case of blackberry, cucumber, and goldenrod, in which 24-methylenecholesterol represented 5% or less of the total sterols, whereas, this sterol accounted for 12.7–35.9% of the sterols from other pollen samples. The levels of sitosterol were quite low (3.1–7.9%) in goldenrod, corn, and dandelion pollens, as was the level of isofucosterol (5.6%) in goldenrod pollen, but these two sterols were present in substantial amounts in all other pollens. The most striking variations in sterol content of these pollen samples occurred in the goldenrod pollen sterols, in which Δ^7-stigmasten-3β-ol was the predominant sterol and in the corn pollen in which $\Delta^{7,24(28)}$-campestadien-3β-ol was the major component. To our knowledge, this is the first report of the former compound as a major sterol of pollen but the latter compound was previously found in corn pollen [9]. Neither cholesterol nor desmosterol was found in all pollen samples, but at least one or the other was identified in all but the maple and corn pollen samples.

TABLE 1.Relative Percentages of Sterols Isolated From Pollen, Adult, and Prepupal Samples Taken From Honeybee Colonies in the Field

Sterol[a]	Pollen	Adults	Prepupae
	Maple colony		
Cholesterol	NI[b]	0.7	0.6
Desmosterol	NI	NI	NI
24-Methylenecholesterol	12.7	41.7	55.8
Sitosterol	18.3	12.7	13.2
Isofucosterol	55.1	22.0	19.0
	Blackberry colony		
Cholesterol	NI	0.8	0.5
Desmosterol	2.9	0.4	0.6
24-Methylenecholesterol	5.1	17.0	43.0
Sitosterol	47.3	38.3	26.0
Isofucosterol	32.4	37.9	16.9
	Cucumber colony		
Cholesterol	0.5	0.6	0.9
Desmosterol	0.7	1.5	2.6
24-Methylenecholesterol	2.7	12.0	37.6
Sitosterol	63.1	47.9	32.2
Isofucosterol	21.2	30.9	14.1
	Goldenrod colony		
Cholesterol	0.6	2.0	1.0
Desmosterol	NI	NI	NI
24-Methylenecholesterol	1.5	34.6	52.6
Sitosterol	5.7	12.4	9.8
Isofucosterol	5.6	21.8	12.6
Δ^7-Stigmasten-3β-ol	66.2	20.4	13.2
	Corn colony		
Cholesterol	NI	0.6	0.6
Desmosterol	NI	0.7	0.3
24-Methylenecholesterol	15.0	27.0	59.5
$\Delta^{7,24(28)}$-campestadien-3β-ol	48.9	1.7	1.5
Sitsosterol	3.1	22.0	11.7
Isofucosterol	23.0	41.9	16.4
	Honeysuckle colony		
Cholesterol	0.3	—[c]	0.4
Desmosterol	NI	-	NI
24-Methylenecholesterol	20.7	-	55.0
Sitosterol	42.9	-	18.6
Isofucosterol	23.1	-	16.1
	Dandelion colony		
Cholesterol	0.4	—	0.6
Desmosterol	NI	-	NI
24-Methylenecholesterol	35.9	-	65.6
Sitosterol	7.9	-	14.0
Isofucosterol	16.9	-	14.2

[a]Other minor sterols not listed in order to facilitate comparison between colonies.
[b]NI = None identifiable.
[c]Adult samples from honeysuckle and dandelion colonies were not collected.

The composition of the pollen sterols was generally reflected in the sterols of the adult bees, except for the corn colony adults. These, as well as the prepupal samples, contained only a small amount of $\Delta^{7,24(28)}$-campestadien-3β-ol. Cholesterol was a minor component in all adult and prepupal honey bee samples, but desmosterol was identified only in samples from the blackberry, cucumber, and corn colonies. In the five adult samples, the levels of 24-methylenecholesterol varied widely (from 12.0% to 41.7%), as did the concentrations of sitosterol (from 12.4% to 47.9%) and isofucosterol (from 20.4% to 41.9%) in these samples. In the prepupal samples, 24-methylenecholesterol was the predominant sterol in every case, and values ranged from 37.6% to 65.6%. Both sitosterol and isofucosterol were present at concentrations of 11.7% or more in all prepupal samples.

DISCUSSION

Comparisons of the pollen, adult, and prepupal sterol samples readily show the ability of the adults, which came from a single commercial source, to provide selectively certain sterols to the developing brood. Although 24-methylenecholesterol is a major sterol of many pollens [10], it was present in very low concentrations in our samples of blackberry, cucumber, and goldernrod pollens, but in high concentrations (43.0%, 37.6%, and 52.6% respectively) in the sterols from prepupal samples from these colonies. This indicates that the adult bees in field colonies are capable of cycling certain sterols from their own endogenous pools, while serving as nurse bees, to make them available to the developing brood via the brood food. Sitosterol and isofucosterol were accumulated by developing larvae in significant quantities under these field conditions even when these sterols were only minor components of the pollen used by each colony. In addition, the developing larvae may have selectively accumulated these sterols directly from pollen and other brood food fed to them.

Further evidence supports the hypothesis that the adults selectively transfer sterols to the developing brood in field colonies much as they do in cage studies with chemically defined diets [1, 4, 5]. For example, in the goldenrod colony adults, Δ^7-stigmasten-3β-ol comprises 20.4% of the sterols, which indicates that this sterol replaced a similar amount of the original adult sterols that were transferred to the brood food while they served as nurse bees (i.e. prior to becoming foragers). The transfer must have been selective, since only 13.2% of the prepupal sterols was Δ^7-stigmasten-3β-ol even though the larval diet included pollen and they could acquire some sterols directly from pollen. Also, 24-methylenecholesterol comprised 52.6% of the goldenrod prepupal sterols, but only 1.5% of goldenrod pollen sterols, and we have previously shown that this sterol is not produced from other sterols in any stage of the honeybee [1]. On the other hand, even though $\Delta^{7,24(28)}$-campestadien-3β-ol represented nearly 49% of the total sterols of corn pollen, the sterols of adult bees and prepupae contained less than 2% of this sterol. These results indicate that Δ^7-stigmasten-3β-ol is selectively incorporated into both the adult and prepupal sterol pools far more readily than is $\Delta^{7,24(28)}$-campestadien-3β-ol.

We previously had found low but identifiable levels of cholesterol and desmosterol in all honeybee samples examined [1, 2, 4]. In the present study we found cholesterol in all prepupal sterol samples, but none was detected in three of the pollen samples. These findings are further evidence for the selective transfer of certain sterols from the endogenous pools of the adults (nurse bees) to the brood food. The presence of either cholesterol or desmosterol in the sterols of prepupae indicates that it was provided by the adult nurse bees, or that it originated from the pollen, since we have shown that honey bees are unable to dealkylate C_{28} or C_{29} phytosterols to produce C_{27} sterols such as cholesterol and desmosterol [1,2].

This study showed that, even under field conditions with different pollen sources, the sterols of honeybee prepupae are comparable to those of prepupae reared under cage conditions with a well-defined artificial diet [1, 4]. It also showed that the mechanism by which adult bees selectively transfer certain sterols from their endogenous pools to the brood is much the same under field conditions and controlled cage conditions. The mechanism is not understood, but may involve the hypopharyngeal glands, mandibular glands, the honey stomach, or some combination of these. Perhaps, analyses of these glands and the honey stomach will provide further insight into this most fascinating aspect of honeybee biochemistry.

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